ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES USING CELLULASE

NASIHAH BT MOHD NOR

UNIVERSITY MALAYSIA PAHANG

ENZYMATIC EXTRACTION OF STEVIOSIDE FROM STEVIA REBAUDIANA LEAVES

NASIHAH BT MOHD NOR

A thesis submitted in fulfilment of the requirement

For the award of the degree of

Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical and Natural Resources Engineering

UNIVERSITY MALAYSIA PAHANG

FEBRUARY 2013

ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* LEAVES BY USING CELLULASE

ABSTRACT

Stevioside is one of the components known as diterpene glycoside existing in *Stevia Rebaudiana* leaves which have 250 to 300 sweeter than sucrose at the concentration of 0.4% (w/v). It is used as sweetening agents and taste modifier mostly in food industry. Moreover, stevioside has no calorific value and suitable used in therapeutic especially in treating diabetic people. The objective of this research is to extract the stevioside from *Stevia rebaudiana* leaves by using cellulase from *Aspergillus Niger*. The medium for enzyme used is acetate buffer while ethanol is applied as solvent in the enzymatic extraction of stevioside by performing various parameters such as concentration of enzyme, incubation time and temperature. Enzymatic extraction method gives the highest concentration of stevioside (900µg/ml) at 50°C as maximum temperature. The concentration of cellulase at 2% (w/v) gives the highest concentration of stevioside (830µg/ml) and the incubation times of 60 minutes gives the maximum time required to complete the extraction process of stevioside. Therefore, it can be concluded that the extraction of stevioside from Stevia rebaudiana leaves using cellulase is a new efficient way of obtaining high concentration of stevioside and also can minimize the use of solvent and energy consumption in degrading the cell wall.

ENZYMATIC EXTRACTION OF STEVIOSIDE FROM *STEVIA REBAUDIANA* BY USING CELLULASE

ABSTRAK

Stevioside adalah salah satu komponen yang dikenali sebagai glikosida diterpene yang wujud dalam daun Stevia rebaudiana yang juga mengandungi kemanisan di antara 250 hingga 300 lebih dari sucrosa (0.4% w/v). Stevioside adaalah agen pemanis dan pengubahusuai rasa dalam industri. Tambahan pula,stevioside tidak mengandungi nilai kalori dan sesuai digunakan untuk tujuan terapeutik terutama merawat penghidap penyakit diabetes. Objektif utama penyelidikan ini adalah untuk mengekstrak stevioside daripada daun *Stevia rebaudiana* dengan menggunakan sellulase daripada *Aspergillus niger*. Medium untuk enzim yang digunakan adalah buffer asetat manakala etanol digunakan sebagai pelarut dalam pengekstrakan enzim stevioside dengan mengendalikan pelbagai parameter seperti kepekatan enzim, masa pengeraman dan suhu. Kaedah pengekstrakan enzim memberikan kepekatan tertinggi stevioside (900μg/ml) pada 50°C sebagai suhu maksimum. Kepekatan sellulase pada 2% (w/v) memberikan kepekatan tertinggi stevioside (830μg/ml) dan masa pengeraman selama 60 minit memberikan masa maksimum yang diperlukan untuk melengkapkan proses pengekstrakan stevioside. Kesimpulannya, pengekstrakan stevioside daripada daun *Stevia Rebaudiana* dengan menggunakan sellulase adalah salah satu cara yang baru dan cekap untuk mendapatkan kepekatan stevioside yang tinggi dan juga boleh mengurangkan penggunaan pelarut yang banyak dan juga tenaga untuk pemecahan dinding sel.

TABLE OF CONTENT

CHAPTER 2 LITERATURE REVIEW

CHAPTER 3 METHODOLOGY

LIST OF FIGURE

LIST OF TABLE

LIST OF SYMBOLS/ ABBREVIATIONS

ºC	Degree Celcius
%	Percentage
w/v	Weight per volume
α	Alpha
β	Beta
mL	Millilitre
nm	Nanometer
M	Molarity
µm	Micrometer
mg	Milligram
g	Gram
µg	Microgram
US	United State

CHAPTER 1

INTRODUCTION

1.1 Background of Study

One of the biggest diseases of world facing today is diabetes. It is approximately 2.6 million of population was diagnosed with diabetes in Malaysia which has been increasing from year to year. Another problem that Malaysia encounters today is obesity which is also called as overweight. This is due to excessive sugar intake in daily life. By performing this study, there will be an opportunity to develop and enlarge the sweetener market by promoting stevioside to these people throughout this country. *Stevia Rebaudiana* is called sugarleaf or sweetleaf because of high level of sweetness found in the leaves. Its ability to sweeten is more than sucrose level which is about 250 to 300 at concentration of 0.4% (w/v).

There are many sweetness components in stevia leaves but the main components are known to be stevioside and Rebaudioside A because of their high producing of sweet taste. The good thing about stevia is that it does not contain caloric value or non-caloric value. It can be part in weight loss management and also to treat diabetic people and people with high blood pressure. At this time, most of stevioside has been extracted by using standard method such as thermal extraction that will take long processing time and producing low efficiency.

1.2 Problem Statement

Basically, people used common method such as thermal extraction which requires solvent and high temperature in order to possess a quality and yield of stevioside. However, this method might take a longer processing time and need high usage of organic solvent which will cause low efficiency of extraction process. Therefore, enzyme extraction method is crucial for green option as to replace this method in order to minimize the usage of organic solvent as well as to reduce the temperature used.

1.3 Research Objective

The objective of this research is to extract stevioside from *Stevia rebaudiana* leaves by using the application of cellulase.

1.4 Scope of Study

In order to complete objectives, the following scopes have been identified:

i. To determine the enzyme concentration on the stevioside yield.

ii. To evaluate the extraction time on the production of stevioside.

iii. To identify the extraction temperature on the production of stevioside.

1.5 Rationale and Significant of Research

The increasing number of diabetic and obesity people in Malaysia is a big concern and should not be taken lightly. This study is important for people who having both diseases and this can help them to create a healthy lifestyle because the stevioside can possibly treat this disease. Hence, the uses of sucrose can be replaced with stevioside as for lowering the sucrose intake.

CHAPTER 2

LITERATURE REVIEW

2.1 Stevia rebaudiana

Stevia rebaudiana (Bertoni) is an herb which can be found in Paraguay and it is classified as one of 154 members of the genus Stevia. This herb is one of only two from genus stevia that producing sweet taste and because of its non-caloric value, it is now proven and has been used in Brazil, Argentina, Paraguay, Korea and Japan. Stevia leaves contain stevioside. Other sweetness components that also present in the leaves are steviolbiodise, rebaudioside A, B, C, D and E as well as dulcoside A (Jan and Geuns, 2003). Table 2.1 shows the physical and solubility of each component from *S.rebaudiana* leaves. The commercialization of *S. rebaudiana* leaves for sweetening and flavouring purposes has been quite fast since first being introduced to Japan. In recent years, about 200 metric tons of purified stevioside and other sweetener products were prepared from about 2,000 metric tons of dried plant leaves for the Japanese market (Kinghorn *et al.* 2002).

Table 2.1 Physical and Solubility Data for Eight Sweet Ent-kaurene Glycoside from Leaves of *S.rebaudiana.*

Compound	Melting Point (°C)	Molecular Weight	Solubility in Water (%)
Stevioside	196-198	804	0.13
Rebaudioside A	242-244	966	0.80
Rebaudioside B	193-195	804	0.10
Rebaudioside C	215-217	958	0.21
Rebaudioside D	283-286	1128	1.00
Rebaudioside E	205-207	966	1.70

(Source: Kinghorn *et al.,* 2002)

Figure 2.1 A specimen of *Stevia Rebaudiana* leaves.

(sources: Kinghorn *et al*., 2002)

2.2 Stevioside

Stevioside is a diterpene glycoside that present in the leaves of *Stevia rebaudiana* which has 250 to 300 times more sweetener than sucrose at concentration of 0.4% (w/v). Stevioside is appeared as white crystalline and colourless powder. Although Stevioside is potentially sweet, it also has an unpleasant taste which has limit used. It was concluded that stevioside and rebaudioside were not cariogenic under the conditions of the study. Stevioside do not seem to present a potential toxicity risk for humans at the low consumption levels used in sweetening (Mcmurtry, 2009).

Table 2.2 Physical Properties of Stevioside

Matrix	Physical Properties of Stevioside
Chemical Abstract Name	Kaur-16-en-18-oic acid, 13-[(2-O-β-D-glucopyranolsyl-β-D-glucopyranoyl)oxy]-, B-D-glucopyranosyl ester, (4α)- (9CI)
Other Names	Ethanophenanthrene, Kaur-16-en-18-oic acid derive.; Stevioside (6CI, 7CI); α-G-Sweet; Steviosin
Molecular Formula	$C_{38}H_{60}O_{18}$
Molecular Weight	804.88
Melting Point	196-198℃

(sources: Kinghorn *et al*., 2002)

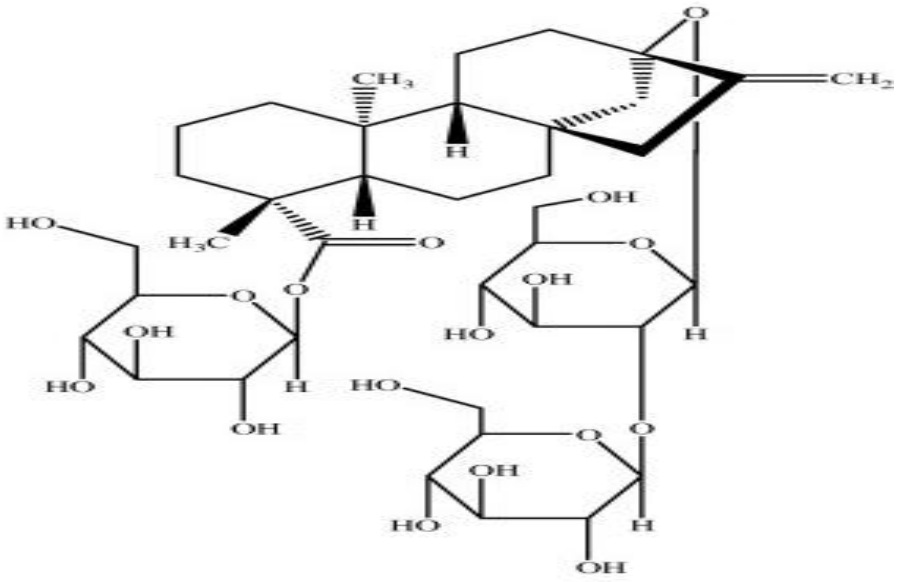

Figure 2.2 Molecular Structures of Steviol and Stevioside in *Stevia Rebaudiana* Leaves.

(Source: Ursula, 2012)

2.2.1 Applications of Stevioside

A refined extract of at least 60 percent of stevioside from the leaves of *S.rebaudiana* and as well as pure stevioside that is free from steviol and isosteviol is approved in Brazil. This stevioside is used for the sweetening of chewing gum, dietetic foods and beverages, medicines, oral hygiene products, and soft drinks. Furthermore, *S. rebaudiana* products are also used as dietary supplements in the United States (US) and other countries such as Italy in Western Europe (Jan & Geuns, 2003)

2.3 Extraction Process

Extraction process is very crucial because of its short time consuming apart from getting the main components of bioactive compounds in plants. The isolations of different types of compounds can proceed by performing the analysis of the product gained using a few equipments such as spectrophotometer, High performance liquid chromatography (HPLC) or gas chromatography (GC) etc. Numerous methods have been used for the bioactive compounds especially the chemical extraction method whereby the chemical used can be obtained easily.

For examples, the mixture of good solvent for antioxidants extraction is acetone and water has been used in order to get the extracts. The high productivity of the extract can be applied by performing the intensification techniques such as ultrasonic waves, supercritical fluids or microwaves were associated with the extraction of plants to improve the yield and quality of extracted products (Wang, 2006). Recently enzyme extraction methods have been reported that the usage of enzyme can increase the product released from plants and therefore, the enzymatic extraction is used as to replace the chemical extraction in order to minimize the use of chemical such as chemical and heat.

2.3.1 Enzymatic Extraction

Selected enzymes can degrade the cell wall by breaking down the structural integrity of the cell wall and increased the solvent accessibility and released the bioactive compounds from intracellular compartments (Pinelo et al., 2008). Examples of enzymes are peptinase, cellulase and xylanase which can disrupt the cell wall of any plants materials. In conventional method, the addition of the enzyme solution to the enzyme extractor affects the carbohydrates compositions, the gallic acid concentration, the acid stability and the cold water solubility and yield. The extractor is preferably temperature controlled as to maximize the effects of the enzymes.

2.3.2 The Advantages of Enzyme Extraction

This method has impressive effects with characteristics of high catalytic efficiency, high specificity, mild reactive conditions and preserving the original efficacy of active compounds to the maximum method. Enzymatic extraction has many advantages, such as shorter time, less solvent, higher extraction rate and better products with lower cost (Meyer & Sowbhagya, 2010).

2.4 Cellulase Enzyme

Fungi are the major production of cellulase although bacteria and actinomycetes also can produce a yield of cellulase. Commercial crude enzyme cellulase from this Fungal like *Aspergillus niger* and *Trichoderma* which are known to be efficient cellulase producers are now can be used in agriculture. This species attack cellulose and produced large amount of cell free cellulase yet still able to hydrolyze cellulose into soluble sugar by fermentation such as glucose (Milala *et al.*, 2009). Cellulose is a linear polymer of anhydroglucose units linked together by b-1,4-glycosidic bonds and found as a major component of plant biomass. The main component of cellulase enzyme is endo-β-D-glucase and catalyzes the hydrolysis of cellulose by tearing apart the sugar residues within the molecules. It is also can convert cellulose into glucose and can be used in industrial scale (Sohail *et al*., 2009).

2.4.1 Preparation of Cellulase

Sohail and co-workers (2009) stated that the commercial cellulase preparation from *Trichoderma reesei* is popular as it contains of both exo-glucanase and endo-glucanase but a low level of β-glucosidase. Hence, *Aspergillus niger* is the most common fungal that widely used in industry as it possesses all three essential components of cellulase system and produced relatively large quantities of endoglucanase and β-glucosidase but low levels of exoglucanase. The production of cellulase was conducted in two methods which are solid state fermentation and submerged fermentation.

A comparison between both methods shown that submerged method has shearing forces deactivated the enzymes, so the enzyme's activity will be decreased. Meanwhile, the production of cellulase from solid state fermentation shown the higher activity of enzyme was produced when agricultural waste was used as the carbon source.